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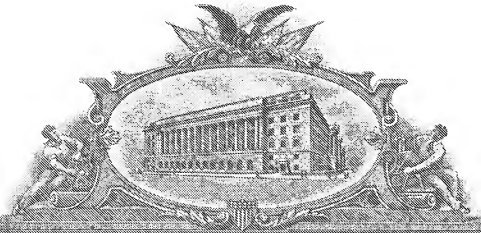

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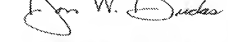

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INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Stefan H.I.	Kappe	Seattle, WA

Additional inventors are being named on the one *separately numbered sheets attached hereto*

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Genetically modified Plasmodium parasites as a protective live-attenuated experimental malaria vaccine

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ The address corresponding to Customer Number:

OR

☑ Firm or Individual Name Thomas F. Broderick Esq.

Address: Seattle Biomedical Research Institute, 307 West Lake Ave N, Suite 500

City: Seattle | State: WA | Zip: 98109-5219

Country: US | Telephone: 206 256 7200 | Fax:

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INVENTOR(S)/APPLICANT(S)

Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Kai	Matuschewski	Heidelberg, Germany

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Genetically modified *Plasmodium* parasites as a protective live-attenuated experimental malaria vaccine

Ann-Kristin Mueller[1], Mehdi Labaied[2], Stefan H. I. Kappe[2,3]* & Kai Matuschewski[1]*

[1]*Department of Parasitology, Heidelberg University School of Medicine, Heidelberg, Germany*
[2]*Seattle Biomedical Research Institute, Seattle, Washington 98109, USA*
[3]*Department of Pathobiology, University of Washington, Seattle, Washington 98195, USA*

*These authors contributed equally to this work

Malaria is a mosquito-borne disease that is transmitted by inoculation of the *Plasmodium* parasite sporozoite stage. Sporozoites invade hepatocytes[1], transform into liver stages, and subsequent liver-stage development ultimately results in release of pathogenic merozoites[2]. Liver stages of the parasite are a prime target for malaria vaccines because they can be completely eliminated by sterilizing immune responses, thereby preventing malarial infection[2]. Using expression profiling, we previously identified genes that are only expressed in the pre-erythrocytic stages of the parasite[4,5]. Here, we show by reverse genetics that one identified gene, *UIS3* (upregulated in infective sporozoites gene 3), is essential for early liver-stage development. *uis3*-deficient sporozoites infect hepatocytes but are unable to establish blood-stage infections *in vivo*, and thus do not lead to disease. Immunization with *uis3*-deficient sporozoites confers complete protection against infectious sporozoite challenge in a rodent malaria model. This protection is sustained and stage specific. Our findings demonstrate that a safe and effective, genetically attenuated whole-organism malaria vaccine is possible.

Malaria has a tremendous impact on human health, killing millions of people annually, and the disease is a major impediment for social and economic development of nations in malaria-endemic areas, particularly in sub-Saharan Africa[6]. Because an effective 'subunit' malaria vaccine has remained elusive, and the complexity of the malaria parasite *Plasmodium* might preclude the successful development of such a vaccine, there has been renewed interest in whole-organism vaccine approaches against malaria[7]. The feasibility of such a vaccine has been demonstrated in animal models and subsequently in humans by the induction of sterile protective

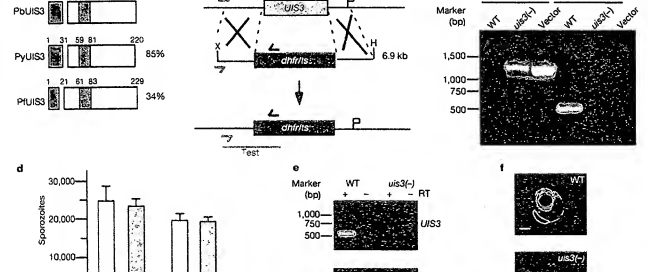

Figure 1 Targeted gene disruption of *P. berghei UIS3*. **a**, Primary structure of *Plasmodium* UIS3 proteins. Predicted cleavable signal peptides and transmembrane spans are boxed in red and blue, respectively. Amino acid sequence identities of the *P. yoelii* and *P. falciparum* UIS3 orthologues (EAA22537 and PF13_0012, respectively) are indicated as percentage of identical residues compared with the *P. berghei* sequence. **b**, Replacement strategy to generate the *uis3*(−) parasite. The wild-type (WT) *UIS3* genomic locus is targeted with an *Eco*RI/*Hin*dIII-linearized replacement plasmid containing the 5′ and 3′ untranslated regions of the *UIS3* open reading frame (ORF) and the *Toxoplasma gondii dhfr/ts* positive selectable marker. Upon a double crossover event the *UIS3* ORF is replaced by the selection marker. Replacement-specific test primer combinations are indicated by arrows, and expected fragments are shown as lines. **c**, Replacement-specific PCR analysis. Confirmation of the predicted gene targeting is achieved by primer combinations that can only amplify a signal from the recombinant locus. Black and grey arrows in **b** indicate primers that hybridize to regions in the plasmid backbone and within or outside the *UIS3* ORF, respectively. A wild-type-specific PCR reaction confirms the absence of residual wild-type parasites in the clonal *uis3*(−) parasite population. **d**, *UIS3* has no function in sporozoite development and salivary gland invasion. Shown are mean numbers (± s.e.m.) of midgut oocyst sporozoites and salivary gland sporozoites at day 14 and day 18 after feeding, respectively. Data are from five independent feeding experiments. **e**, Depletion of *UIS3* transcripts in *uis3*(−) parasites. cDNA from wild-type and *uis3*(−) sporozoites was amplified at 35 PCR cycles. Note the absence of a *UIS3* signal compared to a transcript control (*TRAP*). **f**, Depletion of *UIS3* does not affect sporozoite gliding locomotion. Shown are representative immunofluorescence stainings of wild-type and *uis3*(−) salivary gland sporozoites with an anti-PbCSP antibody[16] that recognizes trails deposited on glass slides. Scale bars, 5 μm.

letters to nature

immunity through inoculation with irradiation-attenuated parasites[8,9]. The recent availability of complete *Plasmodium* genome sequences[10,11] may now permit the development of live-attenuated parasites by more precise and defined genetic manipulations.

Our earlier studies identified *Plasmodium* genes that are specifically expressed during the pre-erythrocytic part of the parasite life cycle[4,5]. A number of pre-erythrocytic genes named *UIS* also undergo upregulation in sporozoites when they gain infectivity for the mammalian host[4]. We reasoned that inactivation of *UIS* genes for which expression is restricted to pre-erythrocytic stages might lead to attenuation of the liver-stage parasite, without affecting the blood stages or mosquito stages. We focused on a gene called *UIS3*, which encodes a small conserved transmembrane protein (Fig. 1a). *UIS3* is expressed in infectious sporozoites[5], and we determined that it is also expressed after sporozoite infection of livers *in vivo* (data not shown). UIS3 of rodent malaria parasites and UIS3 of the human malaria parasite *Plasmodium falciparum* show 34% amino acid sequence identity (Fig. 1a). Because rodent malaria

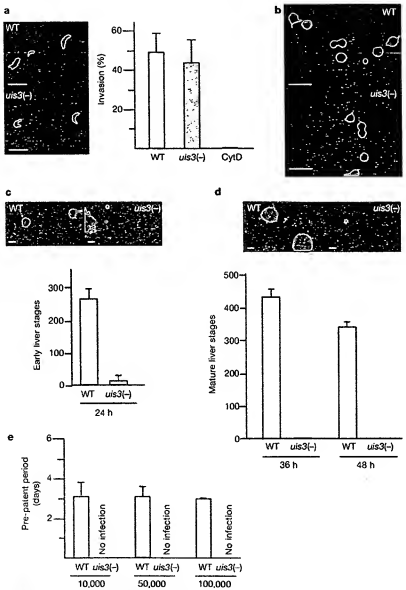

Figure 2 Arrested liver-stage development in *uis3*(−) parasites. **a**, *UIS3* is not required for hepatocyte invasion. Shown are representative double immunofluorescence stains[12] (anti-PbCSP[26]) of cultured hepatoma cells infected with wild-type and *uis3*(−) sporozoites. Extracellular and intracellular sporozoites are labelled red and green, respectively. Quantification of the percentage (± s.e.m.) of invaded sporozoites from three independent experiments is shown to the right. Scale bars, 10 μm. **b**, *UIS3* is not required for initial sporozoite/trophozoite transformation *in vitro*. Wild-type and *uis3*(−) salivary gland sporozoites were added to subconfluent hepatoma cells, and transforming parasites that develop intracellularly were immunostained with anti-HSP70. Shown are representative immunofluorescence stains after 8 h. Scale bars, 10 μm. **c**, *uis3*(−) parasites are impaired in complete transformation into liver-stage trophorozoites. Representative immunofluorescence stains after 24 h are shown. Scale bars, 10 μm. The mean numbers (± s.e.m.) of early liver stages were calculated from three independent experiments each. **d**, *uis3*(−) parasites fail to develop into mature liver-stage schizonts. Representative immunofluorescence stains after 48 h are shown. Scale bars, 10 μm. The mean numbers (± s.e.m.) of mature liver stages after 36 and 48 h were calculated from three independent experiments each. **e**, *uis3*(−) parasites are completely blocked in progression to blood-stage infections in the mammalian host. Wild-type and *uis3*(−) sporozoites were injected intravenously into highly susceptible young Sprague–Dawley rats at the numbers indicated. The occurrence of erythrocytic stages was monitored by daily examination of Giemsa-stained blood films. Experiments were carried out in duplicate with four animals for wild-type and *uis3*(−) parasites, respectively. Error bars indicate s.e.m.

parasites such as *Plasmodium berghei* are excellent models in which to study *Plasmodium* liver-stage and pre-erythrocytic immunity we pursued investigation of *UIS3* in this species.

The endogenous *P. berghei UIS3* gene (*PbUIS3*) was deleted using a replacement strategy[12] (Fig. 1b). After transfection, parental blood-stage parasites were used to obtain clonal parasite lines designated *uis3*(−) that contained exclusively the predicted locus deletion (Fig. 1c). As expected, *uis3*(−) parasites showed normal asexual blood-stage growth and normal transmission to the *Anopheles* mosquito vector (data not shown). Within the mosquito, *uis3*(−) sporozoites developed normally in midgut oocysts and infected the salivary glands in numbers comparable to wild-type sporozoites (Fig. 1d). Polymerase chain reaction with reverse transcriptase (RT–PCR) confirmed lack of *UIS3* expression in *uis3*(−) sporozoites (Fig. 1e). *uis3*(−) sporozoites showed typical gliding motility, a form of substrate-dependant locomotion critical for sporozoite transmission and infectivity[13] (Fig. 1f). They also retained their host-cell invasion capacity of cultured hepatoma cells at levels comparable to wild-type parasites (Fig. 2a).

Intracellular *uis3*(−) sporozoites initiated the typical cellular transformation process that leads to de-differentiation of the banana-shaped, elongated sporozoite to a spherical liver trophozoite[14,15] (Fig. 2b). However, *uis3*(−) parasites showed a severe defect in their ability to complete transformation into liver trophozoites (Fig. 2c). Only a small fraction of *uis3*(−) parasites developed into spherical, early liver stages, and those that did so appeared consistently smaller than the corresponding wild-type forms. Consequently, mutant parasites lacked the capacity to progress to mature liver schizonts (Fig. 2d). On the basis of this extreme developmental defect observed *in vitro*, we next tested whether *uis3*(−) sporozoites had lost their capacity to progress through liver-stage development and cause blood-stage infections *in vivo*. Indeed, intravenous injection of up to 100,000 *uis3*(−) sporozoites failed to induce blood-stage parasitaemia in young Sprague–Dawley rats, which are highly susceptible to *P. berghei* sporozoite infections (Fig. 2e). Control wild-type sporozoites induced blood-stage parasitaemia in rats between 3–4 days after injection.

Thus, the observed phenotypic characteristics of *uis3*(−) parasites (that is, their ability to invade hepatocytes and their defect in complete liver-stage development) allowed us to test them as a whole-organism vaccine in a mouse–sporozoite challenge model. We intravenously immunized mice with *uis3*(−) sporozoites using different prime–boost regimens, and subsequently challenged the mice by intravenous injection of infectious wild-type sporozoites (Table 1). Protection was evaluated by blood smear to detect the development of blood-stage parasitaemia starting 2 days after sporozoite challenge—the most stringent readout for sterile protection against malarial infection. Priming with 50,000 *uis3*(−) sporozoites followed by two boosts with 25,000 *uis3*(−) sporozoites completely protected all immunized mice against a challenge with 10,000 wild-type sporozoites administered 7 days after the last boost (Table 1). Complete sterile protection against the same sporozoite challenge dose was also achieved with a similar prime–two-boost protocol using 10,000 *uis3*(−) sporozoites (Table 1). We next immunized mice using the same prime–boost protocols but challenged them with wild-type sporozoites 4 weeks after the last boost. None of the challenged mice developed blood-stage infections and thus enjoyed protracted sterile protection (Table 1). Protracted protection was confirmed by a re-challenge experiment where protected animals were challenged again with a high inoculum of 50,000 infectious sporozoites after 2 months. All animals remained completely protected. Mice immunized with *uis3*(−) sporozoites were also completely protected against re-challenge by infectious mosquito bite (Table 1). To determine the level of protection with a reduced immunization dose, we tested a prime–single-boost protocol with 10,000 *uis3*(−) sporozoites. Seven out of ten animals enjoyed complete protection, whereas the remaining three animals became patent after a long delay in patency. Next, a subset of immunized mice was challenged by direct inoculation with blood-stage parasites. All animals developed blood-stage parasitaemia two days after challenge, indicating that the observed protective immunity is not acting against blood stages and thus is specific against pre-erythrocytic stages. Finally, to evaluate a more vaccine-relevant delivery route we immunized mice subcutaneously using a prime–two-boost protocol with 50,000 *uis3*(−) and 25,000 *uis3*(−) sporozoites, respectively. All mice were completely protected against subsequent intravenous wild-type sporozoite challenge.

Our results show that it is possible to develop genetically modified malaria parasites that are completely attenuated at the liver stage—the stage at which infection of the mammalian host after mosquito transmission is normally established. This attenuation was achieved by deletion of a single parasite gene, *UIS3*. Although UIS3 function remains unknown, *uis3*(−) parasites clearly lacked the ability to compensate for its loss. The protracted sterile protection against malaria that we observed after immunization with *uis3*(−) sporozoites in the mouse–sporozoite challenge model provides proof of principle that a genetically modified malaria vaccine is feasible. We identified a *UIS3* orthologue in the genome of the most lethal human malaria parasite, *P. falciparum*. This will allow us to create a genetically attenuated *uis3*(−) human parasite that can be tested as a vaccine in human–sporozoite challenge models. Together, our findings lead the way to the development of a genetically attenuated, protective whole-organism malaria vaccine that prevents natural infection by mosquito bite.□

Table 1 Protection of C57Bl/6 immunized mice against challenge with wild-type *P. berghei* sporozoites

Experiment	Immunization (*uis3*(−) sporozoites)	Boosts*	Challenge dose (time point)†	Number protected/number challenged (pre-patency)†
1	50,000	25,000 (d 14)/25,000 (d 21)	10,000 sporozoites (d 7)	10/10 (no infection)§
1	10,000	10,000 (d 14)/10,000 (d 21)	10,000 sporozoites (d 7)	10/10 (no infection)§
1	–	–	10,000 sporozoites	0/9 (d 3)
2	50,000	25,000 (d 34)/25,000 (d 45)	10,000 sporozoites (d 30)	5/5 (no infection)
2	10,000	10,000 (d 34)/10,000 (d 45)	10,000 sporozoites (d 30)	5/5 (no infection)
2	–	–	10,000 sporozoites	0/5 (d 4.5)
3	50,000	50,000 (d 14)/10,000 (d 21)	Ten infectious mosquitoes (d 38)	5/5 (no infection)
3	10,000	10,000 (d 14)/10,000 (d 21)	Ten infectious mosquitoes (d 38)	5/5 (no infection)
3	–	–	Ten infectious mosquitoes	0/5 (d 3)
4	10,000	10,000 (d 14)/–	10,000 sporozoites (d 7)	7/10 (d 8)
4	–	–	10,000 sporozoites	0/5 (d 3)
5	50,000	25,000 (d 14)/25,000 (d 21)	10,000 blood stage (d 30)	0/5 (d 2)
5	10,000	10,000 (d 14)/10,000 (d 21)	10,000 blood stage (d 30)	0/5 (d 2)
5	–	–	10,000 blood stage	0/3 (d 2.5)

Mice were immunized with *P. berghei uis3*(−) sporozoites.
*Data are presented as numbers of sporozoites for first boost/second boost. Day of boost is indicated in parentheses.
†Mice were challenged with infectious *P. berghei* wild-type sporozoites or blood stages. Mice were from the same age group (50–80 days old) and sporozoites were from the same mosquito batch. Time points indicate the day of challenge after the final boost.
‡The pre-patent period is defined as the time until the first appearance of a single erythrocytic stage in Giemsa-stained blood smears.
§Five mice of the group were re-challenged with one dose of 50,000 wild-type sporozoites 2 months after the first challenge and remained protected.

letters to nature

Methods

Plasmodium berghei transfection

For replacement of *Pbuis3* two fragments were amplified using primers UIS3rep1for (5′-GGGTACCCGGCATTAGCATAACATCTCATTGG-3′) and UIS3rep2rev (5′-CAAGCTTGCTTTCATATATTTGTTATTTGTC-3′) for the 800-base pair (bp) 3′ fragment, and UIS3rep3for (5′-CGAATTCCCATATGTTTGTGTAAACATC-3′) and UIS3rep4rev (5′-CTCTAGAGGTGTCGCTTAATGTTTGTTTAAAC-3′) for the 760-bp 5′ fragment using *P. berghei* genomic DNA as template. Cloning into the *P. berghei* targeting vector[42] resulted in plasmid pAKM19. To obtain clonal parasite populations, limited dilution series and intravenous injection of one parasite into 15 recipient NMRI mice each was performed. For RT–PCR analysis we dissected 6 × 10^5 *uis3*(−) and 6 × 10^5 wild-type salivary gland sporozoites and isolated poly(A)$^+$ RNA using oligo dT-columns (Invitrogen). For complementary DNA synthesis and amplification we performed a two-step PCR using random decamer primers (Ambion) and subsequent standard PCR reactions.

Phenotypical analysis of *uis3*(−) parasites

Anopheles stephensi mosquito rearing and maintenance was carried out under a 14 h light/10 h dark cycle, 75% humidity and at 28 °C or 20 °C, respectively. For each experiment, mosquitoes were allowed to take a blood meal for 15 min from anaesthetized NMRI mice that had been infected with wild-type *P. berghei* NK65 or the *uis3*(−) clone, and were assayed for a high proportion of differentiated gametocytes and microgametocyte-stage parasites capable of exflagellation. Mosquitoes were dissected at days 10, 14 and 17 to determine infectivity, midgut sporozoite and salivary gland sporozoite numbers, respectively. For analysis of sporozoite motility, sporozoites were deposited onto pre-coated (3% BSA/RPMI 1640) glass coverslips, fixed for 10 min at room temperature with 4% paraformaldehyde, and incubated using primary antibody against *P. berghei* circumsporozoite protein (anti-PbCSP)[36]. To detect liver stages in hepatocytes, ~10^5 Huh7 cells were seeded in eight-chamber slides and grown to semiconfluency. *Plasmodium berghei* sporozoites were added, incubated for 90 min at 37 °C, and washed off. After 8, 12, 15, 24, 36 and 48 h, LS were revealed using primary antibodies against the *P. berghei* heat-shock protein 70 (HSP70)[43]. To analyse sporozoite invasion a double-staining protocol with anti-PbCSP antibody was used[44]. To determine the infectivity of clonal sporozoite populations *in vivo*, young Sprague–Dawley rats were injected intravenously with 100 µl sporozoite suspension in RPMI 1640. Parasitaemia of the animals was checked daily by Giemsa-stained blood smears. The appearance of a single erythrocytic stage represents the first day of patency.

Immunization and parasite challenge experiments

For all experiments female C57BL/6 mice (Charles River Laboratories) at the age of 50–80 days were used. For immunization, *uis3*(−) sporozoites were extracted from the salivary glands of infected mosquitoes. Typically, a single infected mosquito contained 20,000 *uis3*(−) sporozoites. Sporozoites were injected in a volume of 100 µl intravenously into the tail vein or subcutaneously into the neck of animals. Animals were immunized with a single dose of 1 or 5 × 10^4 *uis3*(−) sporozoites, followed by two boosts of either 1 or 2.5 × 10^4 *uis3*(−) sporozoites administered intravenously or subcutaneously. The first boost was given 14 days after the immunization, with a second boost following 7 days thereafter, or at time intervals indicated. One set of animals was immunized followed by a single boost with 1 × 10^5 *uis3*(−) sporozoites each. The animals were then monitored for the parasitaemia by daily blood smears. All animals remained negative for the parasite blood stage after the first immunization and subsequent boosts. Animals were challenged 7 days and up to 1 month after receiving the last boost of *uis3*(−) sporozoites by intravenous or subcutaneous injection of either 5 × 10^4 or 1 × 10^5 infectious *P. berghei* wild-type sporozoites. For each set of experiments at least three naive animals of the same age group were included to verify infectivity of the sporozoite challenge dose. In each naive animal, parasitaemia was readily detectable by Giemsa-stained blood smears at days three–five after injection. Protected animals were monitored for at least 14 days and typically up to 1 month. A re-challenge study was performed for one immunization experiment. 2 months after the first challenge, with a single dose of 5 × 10^4 infective *P. berghei* wild-type sporozoites. To test whether *uis3*(−) immunized mice were protected against re-challenge by natural transmission, ten protected and five naive control mice were exposed for 10 min to ten highly infected mosquitoes that contained an average of 60,000 wild-type salivary gland sporozoites each. Successful blood feeding was confirmed by mosquito dissection after the challenge experiment. To confirm stage specificity of protection, an additional experiment was performed with ten mice that were fully protected against a challenge with infectious sporozoites. All immunized mice and three naive control mice were challenged by intravenous injection of 5 × 10^4 *P. berghei* wild-type blood-stage parasites. All mice were fully susceptible to blood-stage inoculations with no differences in patency.

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Correspondence and requests for materials should be addressed to S.H.I.K. (Stefan.Kappe@sbri.org) or K.M. (Kai_Matuschewski@med.uni-heidelberg.de).